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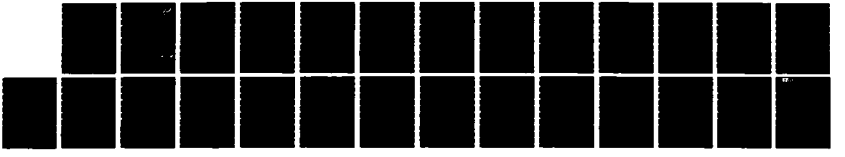

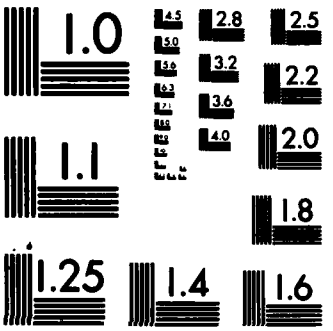

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MONOCLONAL ANTIBODY TESTING OF LYMPHOCYTES AFTER OVERNIGHT STORAGE

by

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Often monoclonal antibody testing of lymphocytes is not performed until the day after blood collection for reasons of convenience or due to the need to transport the blood to other facilities. In order to determine whether accurate results can be obtained on the day after collection, we compared results obtained after storage overnight at 4 C or 22 C with results obtained with fresh lymphocytes. Lymphocytes from 24 normal individuals were evaluated with ten monoclonal antibodies using an immunofluorescence

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technique with analysis by flow cytofluorometry. There were markedly altered results obtained with lymphocytes separated on the day after collection from whole blood stored at 4 C. Lymphocytes separated from whole blood stored at 22 C showed moderate changes in reactivity with some monoclonal antibodies. Lymphocytes that were separated from fresh blood and then stored at 2 C or 22 C showed results similar to fresh lymphocytes. These results underscore the importance of proper processing of blood samples to avoid misinterpretation of results.

ABSTRACT

Often monoclonal antibody testing of lymphocytes is not performed until the day after blood collection for reasons of convenience or due to the need to transport the blood to other facilities. In order to determine whether accurate results can be obtained on the day after collection, we compared results obtained after storage overnight at 4 C or 22 C with results obtained with fresh lymphocytes. Lymphocytes from 24 normal individuals were evaluated with ten monoclonal antibodies using an immunofluorescence technique with analysis by flow cytofluorometry. There were markedly altered results obtained with lymphocytes separated on the day after collection from whole blood stored at 4 C. Lymphocytes separated from whole blood stored at 22 C showed moderate changes in reactivity with some monoclonal antibodies. Lymphocytes that were separated from fresh blood and then stored at 4 C or 22 C showed results similar to fresh lymphocytes. These results underscore the importance of proper processing of blood samples to avoid misinterpretation of results.

INTRODUCTION

The development of monoclonal antibodies directed against unique surface markers on human lymphocyte subsets permits the identification of lymphocyte patterns in health and disease. Monoclonal antibodies have been used for a variety of purposes such as immunologic monitoring of renal transplant patients (1), categorization of leukemia (2), and evaluation of T lymphocyte subpopulations in disease states such as acquired immunodeficiency syndrome (AIDS) (3,4).

Immediate testing of lymphocytes with monoclonal antibodies may not always be possible when blood is collected at odd times of the day or when the blood must be transported to other facilities for evaluation. Often, the lymphocytes are not tested until the day after collection. The question arises whether the values obtained by monoclonal antibody testing of lymphocytes after overnight storage accurately reflect the subpopulations present in fresh blood. The best conditions for overnight storage or shipment of blood samples for monoclonal antibody testing of lymphocytes must be established.

In the study presented here, we have compared results of monoclonal antibody testing of fresh lymphocytes with results obtained after overnight storage of whole blood or separated lymphocytes at room temperature (22 C) and 4 C. There were some marked alterations in reactivity of lymphocytes separated from whole blood stored at 4 C and some moderate alterations in reactivity of lymphocytes separated from whole blood stored at room

temperature (22 C). Lymphocytes that were separated from whole blood prior to storage at 4 C or 22 C showed little or no change in reactivity with ten monoclonal antibodies.

MATERIALS AND METHODS

Peripheral blood from 24 healthy volunteer donors was collected in heparin or ACD anticoagulant. Half of the blood was diluted with phosphate buffered saline (PBS) and the mononuclear cells were separated by ficoll-hypaque density gradient centrifugation. After washing with PBS, a portion of the mononuclear cells was tested immediately with monoclonal antibodies. The remaining mononuclear cells suspended in 0.5% bovine serum albumin (BSA) in PBS were stored overnight at 4 C or 22 C in polypropylene tubes. Half of the original whole blood was stored overnight undisturbed at 4 C or 22 C and on the following day the mononuclear cells were separated using ficoll-hypaque. Thus, the mononuclear cells were stored both in whole blood and as separated cells in buffered saline prior to testing with monoclonal antibodies the following day.

Commercial antibodies were selected to evaluate a wide range of antigenic specifities of T cells, B cells, and null cells: sheep rosette receptor (CCT11)[a] (5); pan-T cell antigen (OKT3)[b] (6); helper/inducer T lymphocytes (CCT4)[a] (7); suppressor/cytotoxic T lymphocytes (CCT8)[a] (8); activated or proliferating cells (OKT9)[b] (9); immature cells, activated T cells, null cells (OKT10)[b] (10,11); B cells (CCB1)[a] (12); HLA D/DR antigens (CCI2)[a] (12); null cells (OKM1)[b] (11,14); granular lymphocytes, natural killer/killer cells (Leu-7)[c] (11,15). For each test, 10^6 mononuclear cells were incubated with 100 ul of appropriately diluted monoclonal antibody on ice for 30 minutes. After washing, FITC conjugated F(ab')2 fragment goat anti-mouse immunoglobulin was added and incubated on ice for

30 minutes. Following subsequent washing, the cells were fixed in 1% formalin in buffered saline.

The cells were analyzed by flow cytofluorometry using the Coulter Epics V system. Viable lymphocytes were distinguished from monocytes, nonviable cells, and erythrocytes on the basis of forward light scatter and 90 degree light scatter. Antibody reactivity is represented by the percentage of 10,000 lymphocytes that demonstrated fluorescence greater than negative control lymphocytes which were incubated with 1% BSA in PBS in place of monoclonal antibody.

RESULTS

Blood from 24 individuals was tested on the day of collection and after overnight storage at 4 C of both separated lymphocytes and whole blood. Results of monoclonal antibody testing are shown in Table 1. Lymphocytes that were separated on the day of collection using ficoll-hypaque and then stored overnight at 4 C showed no significant difference in reactivity with any of the ten monoclonal antibodies. The lymphocytes that were separated from whole blood after overnight storage at 4 C showed marked reduction in reactivity with CCT11, OKT3, and CCT4 monoclonal antibodies with mean differences of -26, -33, and -21, respectively. These lymphocytes showed increased reactivity ranging from 2-15% with OKT9, OKT10, CCI2, OKM1 and Leu-7 antibodies. Only the reactivity with CCT8 and CCB1 antibodies remained unchanged.

TABLE 1

Blood from 9 of the 24 individuals was stored at room temperature (22 C) in addition to 4 C. Results from monoclonal antibody testing after storage at 22 C are shown in Table 2. Lymphocytes that were separated on the day of collection and then stored overnight at 22 C showed mean differences in reactivity with the ten antibodies ranging from 1-8% when compared with fresh lymphocytes. With some monoclonal antibodies, e.g., CCT11, there was a consistent but small change in reactivity which was significant by paired t analysis but which is of no practical importance with regard to interpretation. Duplicate samples usually agree within 1-5% when tested with CCT11 antibody.

TABLE 2

Lymphocytes that were separated from whole blood after storage at

22 C (Table 2) showed a moderate decrease in reactivity with CCT11, OKT3 and CCT4 with mean differences of -13, -18 and -11, respectively. There were mean differences in reactivity with CCI2 and OKM1 of +3 and +6; other differences in reactivity ranged from 1-5% and were not statistically significant.

Differences in reactivity of lymphocytes upon storage varied from person to person. The anticoagulant used (ACD or heparin) did not appear to affect the results, and the data was combined in Tables 1 and 2. There was, however, considerable clumping of platelets and leukocytes in heparinized whole blood kept at 4 C which caused a low yield of mononuclear cells after ficoll-hypaque centrifugation.

The quality of the fluorescence histograms obtained with lymphocytes from stored whole blood was inferior to that of fresh lymphocytes or lymphocytes that were separated prior to storage. Examples of histograms
FIG. 1 comparing CCT4 and negative control fluorescence are shown in Figure 1. Fresh lymphocytes and lymphocytes that were separated prior to storage at either 4 C or 22 C showed a single symmetrical peak in the negative control samples and distinct positive and negative peaks in the samples with CCT4 antibody. Lymphocytes from whole blood stored at 22 C showed a wider peak of fluorescence in the negative control but a distinctly positive peak with CCT4 antibody. Lymphocytes from whole blood stored at 4 C showed an increased percentage of weakly fluorescent cells which altered the shape of the negative control and the CCT4 histograms.

The percent reactivity of lymphocytes with CCT8 antibody was not altered by stora[illegible] However, the quality of the fluorescence histograms

FIG. 2 obtained was variable, as shown in Figure 2. The distinct peak of strongly fluorescent cells is not present with lymphocytes separated from whole blood after storage.

Changes in percent reactivity with the antibody to the sheep rosette receptor (CCT11) and with the pan-T cell antibody (OKT3) are illustrated in Figures 3 and 4. Again, the percent of positive cells is reduced in lymphocytes separated from whole blood after overnight storage.

FIG. 3
FIG. 4

DISCUSSION

These observations on reactivity of monoclonal antibodies with lymphocyte surface antigens can be compared with previous studies on HLA typing and in vitro functional tests of stored lymphocytes. In general, previous reports indicate that while it is sometimes acceptable to store whole blood at room temperature for a short time it is best to separate the lymphocytes from whole blood prior to storage at room temperature. Lymphocytes separated from fresh blood and stored in culture media at room temperature can be accurately tested for HLA A and B locus antigens for 2 to 3 weeks (16-18) although C locus antigens disappear from cells kept 7 days at room temperature (19). Viability of the lymphocytes in culture media was maintained better with storage at room temperature (20-25 C) than at 4 C or 37 C (17,18). Mitogen and antigen responsiveness of lymphocytes from whole blood shipped on ice was diminished in comparison to lymphocytes from whole blood transported at ambient temperature and tested 9-11 hours after collection (20). Others (21) reported that lymphocytes separated from fresh blood and stored at room temperature or 37 C were more responsive to PHA stimulation than lymphocytes stored at 4 C for 72 hours. Lymphocytes obtained from heparinized whole blood stored for 72 hours at room temperature were the least responsive to stimulation (21).

Grunow et al (22) and Naess and Tondur (23) reported a decrease in the number of rosette-forming cells recovered from whole blood stored for 24 hours at 4 C. This paralleled a relative increase in the percentage of B cells as determined by surface immunoglobulin staining. Lymphocytes that

were separated with ficoll-hypaque prior to storage at 4 C showed no change in percentage of T or B cells for up to 48 hours (22). When testing lymphocytes obtained from whole blood stored at 4 C, we observed a similar decrease in T cells detected by reactivity with monoclonal antibodies. This appeared to be due to selective reduction of the helper/inducer T cell subpopulation. There was little or no change in B cells detected by monoclonal antibody CCB1 which is specific for B1 antigen present on B lymphocytes and not monocytes (12). The higher percentage of B cells reported by others (22) using immunoglobulin staining may be due to monocyte contamination. We also observed an increase in reactivity of those monoclonal antibodies that react with null cells or large granular lymphocytes--CCI2, OKT10, OKM1 and Leu-7. These changes in reactivity occurred only when the lymphocytes were separated from whole blood after overnight storage at 4 C. Lymphocytes separated from whole blood after storage at room temperature (22 C) showed moderate changes in reactivity of some monoclonal antibodies. Lymphocytes that were separated from fresh blood and then stored at 4 C or 22 C showed little or no change in reactivity with any of the monoclonal antibodies.

This study was designed to determine whether monoclonal antibody testing of stored lymphocytes is an accurate reflection of the reactivity of fresh lymphocytes. It was not intended as an evaluation of changes in the survival of lymphocyte subpopulations during storage. With fresh blood, about 30-70% of the mononuclear cells are recovered after ficoll-hypaque separation and washing and it is assumed that there is no selective loss of cells. After whole blood is stored overnight, a lower number of the mono-

nuclear cells are recovered. Thus, alterations in the cells (e.g. changes in cell density) during overnight storage could result in selective loss of some subpopulations during the separation procedure. Alternatively, modifications in the cell membrane during storage may result in more or less exposure of surface antigens which react with the monoclonal antibody. Whatever the changes are that occur during storage of whole blood, lymphocytes isolated prior to storage do not appear to be affected. Storage in buffered saline with a small amount of albumin appears to be adequate, although enriched culture medium would also seem appropriate.

In conclusion, our study indicates that significantly altered results are obtained with monoclonal antibody testing of lymphocytes from whole blood kept overnight at 4 C. The alteration in results with T cell antibodies was such that truly normal individuals appeared to have a decreased percentage of T lymphocytes, decreased helper T lymphocytes, and an altered helper/suppressor ratio. While only moderate changes are observed when whole blood is kept at room temperature, we recommend that lymphocytes be separated from fresh whole blood prior to storage or transportation for monoclonal antibody testing.

FIGURE 1

Reactivity of CCT4 monoclonal antibody with (A) fresh lymphocytes, (B) lymphocytes separated from fresh blood and stored at 22C, (c) lymphocytes separated from fresh blood and stored at 4 C, (D) lymphocytes separated from whole blood stored overnight at 22 C, (E) lymphocytes separated from whole blood stored overnight at 4 C.

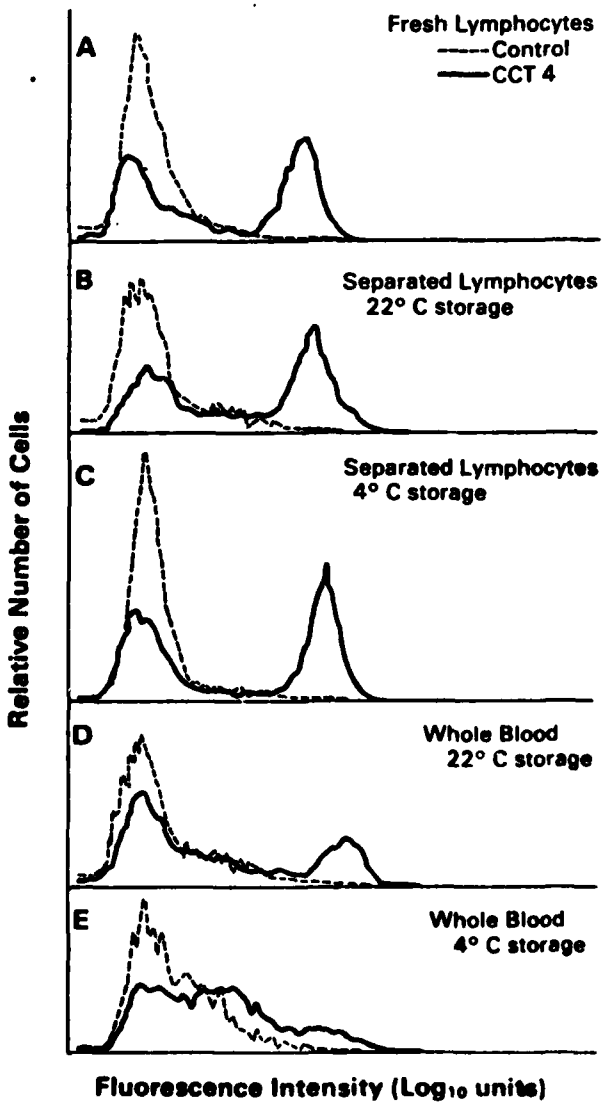

FIGURE 2

Reactivity of CCT8 monoclonal antibody with (A) fresh lymphocytes, (B) lymphocytes separated from fresh blood and stored at 22 C, (C) lymphocytes separated from fresh blood and stored at 4 C, (D) lymphocytes separated from whole blood stored overnight at 22 C, (E) lymphocytes separated from whole blood stored overnight at 4 C.

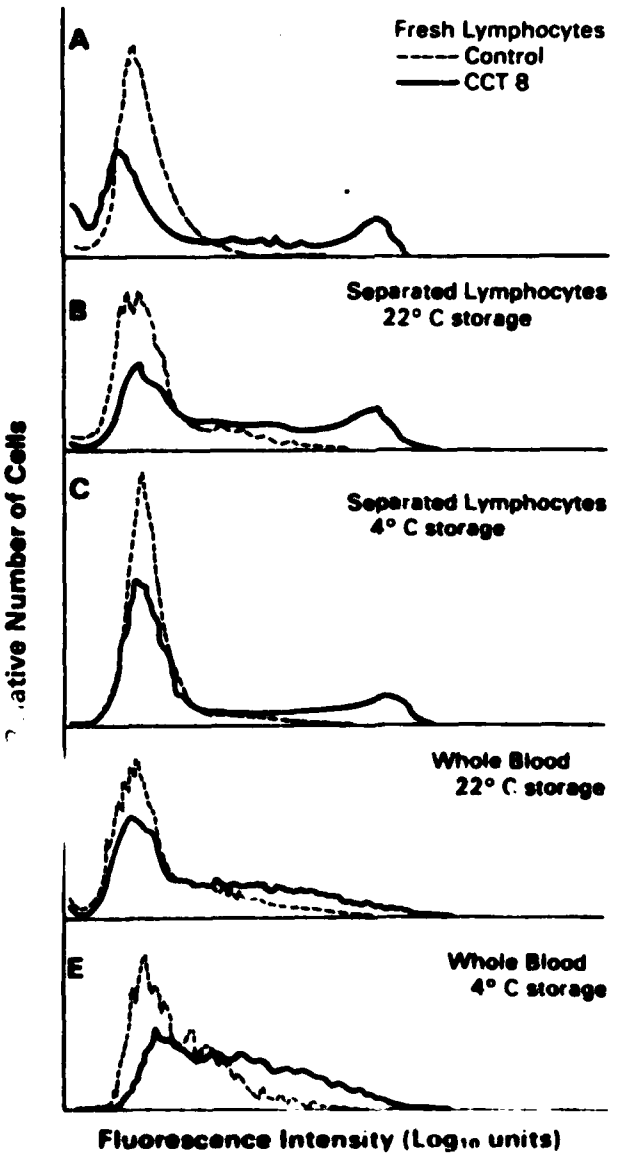

FIGURE 3

Changes in percent reactivity with the antibody to the sheep rosette receptor (CCT11).

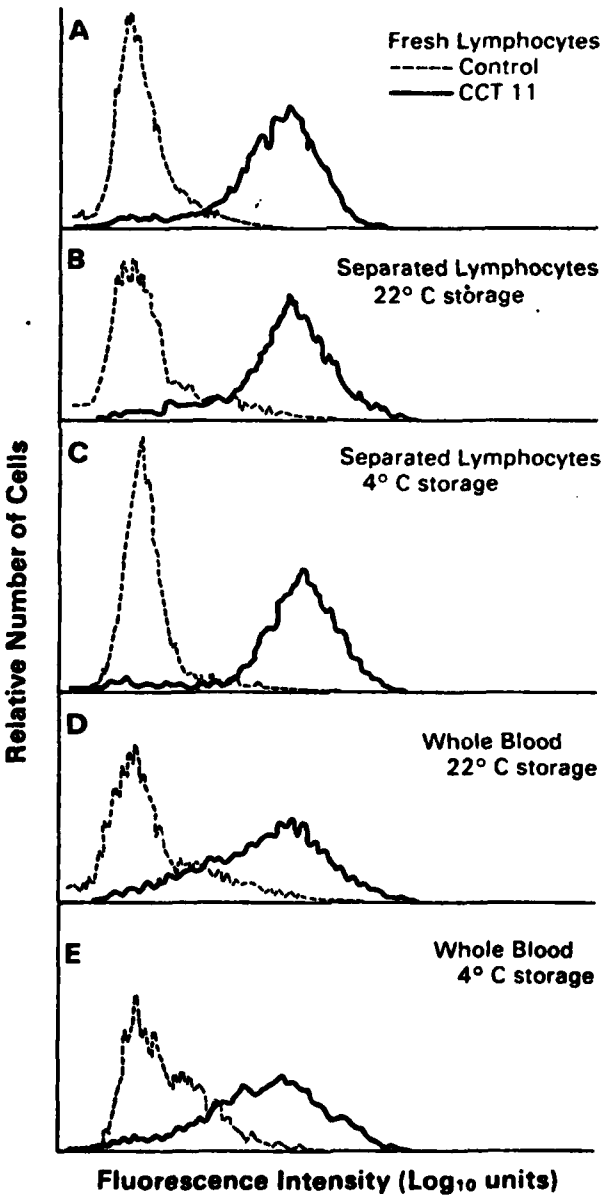

FIGURE 4

Changes in percent reactivity with the antibody to the pan-T cell antibody (OKT3).

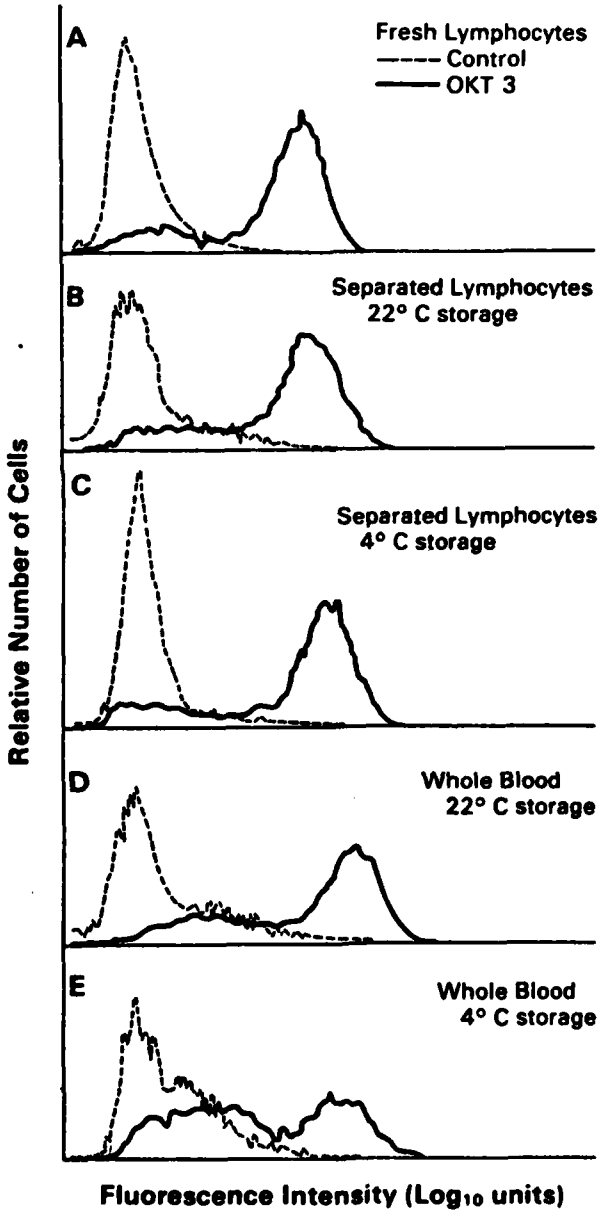

TABLE 1

Percent Reactivity (Mean ± S.D.) of Ten Monoclonal Antibodies with Fresh Lymphocytes, Lymphocytes Separated from Fresh Blood and then Stored at 4 C, and Lymphocytes obtained from Whole Blood After Overnight Storage at 4 C (n = 24)

Antibody	Fresh Lymphocytes	Lymphocytes Stored at 4 C	Whole Blood Stored at 4 C
CCT11	80 ± 8	78 ± 9	54 ± 16**
OKT3	69 ± 12	68 ± 11	36 ± 15**
CCT4	44 ± 8	44 ± 6	23 ± 10**
CCT8	24 ± 6	26 ± 6	22 ± 8
OKT9	3 ± 4	3 ± 4	5 ± 6*
OKT10	12 ± 8	12 ± 7	19 ± 14**
CCB1	9 ± 8	9 ± 9	8 ± 5
CCI2	6 ± 3	6 ± 2	11 ± 5**
OKM1	13 ± 8	17 ± 9	28 ± 17**
Leu-7	9 ± 6	10 ± 5	15 ± 9**

*Significantly different by paired t analysis ($p < 0.05$)
**Significantly different by paired t analysis ($p < 0.01$)

TABLE 2

Percent Reactivity (Mean ± S.D.) of Ten Monoclonal Antibodies with Fresh Lymphocytes, Lymphocytes Separated from Fresh Blood and Stored at 22 C, and Lymphocytes Obtained from Whole Blood after Overnight Storage at Room Temperature (22 C) (n = 9)

Antibody	Fresh Lymphocytes	Lymphocytes Stored at 22 C	Whole Blood Stored at 22 C
CCT11	83 ± 6	80 ± 6*	70 ± 13**
OKT3	77 ± 10	73 ± 8*	59 ± 14**
CCT4	44 ± 7	49 ± 9*	33 ± 11*
CCT8	24 ± 8	28 ± 7*	23 ± 9
OKT9	2 ± 1	5 ± 5	4 ± 3
OKT10	9 ± 5	11 ± 7	13 ± 5
CCB1	14 ± 10	17 ± 13	9 ± 8
CCI2	6 ± 3	7 ± 4	9 ± 2*
OKM1	8 ± 5	16 ± 2*	14 ± 5**
Leu-7	8 ± 4	9 ± 5	9 ± 4

*Significantly different by paired t analysis ($p < 0.05$)
**Significantly different by paired t analysis ($p < 0.01$)

FOOTNOTES

[a]Coulter Immunology

[b]Ortho Diagnostics

[c]Becton-Dickinson

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